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EFFECTS OF ADENOSINE RECEPTOR AGONISTS ON NITRIC OXIDE RELEASE IN MOUSE DURING ENDOTOXEMIA

WEI MIN HON, HOON ENG KHOO, SING SHANG NGOI* and SHABBIR MOOCHHALA*†

Departments of Biochemistry and *Surgery, National University of Singapore, Singapore 0511

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Abstract—The effects of adenosine receptor agonists on plasma NO_x^- (NO_2^- and NO_3^-) production in mice treated with lipopolysaccharide (LPS) were investigated. NO_x^- is the stable decomposition product of nitric oxide (NO), which can be measured as a marker of NO production. Injection of the mice with LPS resulted in increased plasma NO_x^- concentration, reaching a peak after 8 hr (38 times basal level) and then declining slowly. Pretreatment with the adenosine agonists *R*-phenylisopropyladenosine (*R*-PIA), 5′-*N*-ethylcarboxamidoadenosine (NECA), 5′-(*N*-cyclopropyl)carboxamidoadenosine (CPCA) and N^6-cyclohexyladenosine (CHA) 1 hr before LPS administration caused a dose-dependent reduction of plasma NO_x^- concentration. The rank order of inhibitory potency was NECA ≥ *R*-PIA > CPCA > CHA, which is characteristic of neither A_1 nor A_2 receptors.

Key words: adenosine agonist; endotoxemia; nitric oxide; lipopolysaccharide

Recent studies have linked the production of NO‡ to LPS-induced hypotension, vascular hyporesponsiveness and death, suggesting that excess generation of NO plays an important role in the development of septic shock [1, 2]. NO is derived from the oxidation of the terminal guanidino nitrogen atom of L-arginine by NOS, of which two general types have been identified [3]. One is constitutive (cNOS), Ca^{2+}/calmodulin and NADPH dependent. The other, inducible (iNOS) by LPS and cytokines, such as TNF and IL-1, is also NADPH dependent but Ca^{2+} independent. The cNOS is present mainly in vascular endothelium, brain and platelets. The iNOS can be induced in many cells including macrophages, neutrophils, Kupffer cells, hepatocytes, vascular smooth muscle cells and endothelial cells. Once expressed, it catalyses the generation of large quantities of NO, which is cytostatic/cytotoxic to pathogens and tumour cells [4]. Administration of LPS to animals has been shown to result in an increase in the levels of serum nitrate (NO_3^-) and nitrite (NO_2^-), metabolites of NO [5]. This can be inhibited by L-N^G-monomethyl-arginine (L-NMMA), a specific inhibitor of NOS. L-NMMA can also partially overcome the hypotension in septic shock [6].

Recently, a number of reviews have reported the anti-inflammatory properties of endogenous adenosine and its agonists [7], as well as agents that indirectly augment extracellular adenosine concentration [8]. Adenosine regulates various physiological activities by binding to at least two different cellular surface receptors, A_1 and A_2 [9]. *In vitro*, adenosine and its agonists reduce the adhesion of polymorphonuclear leukocytes (PMNs) by occupying A_2 receptors [10] and inhibit human monocyte TNF production [11]. *In vivo*, these agents reduce serum TNF levels in LPS-treated rats [11].

In the present study, the effects of adenosine agonists on the induction of NOS by LPS, as indicated by systemic NO_x^- ($NO_2^- + NO_3^-$) levels, the stable end products of NO oxidation [12], were demonstrated. The anti-inflammatory properties of adenosine may be mediated, in part, via the down-regulation of NO production.

MATERIALS AND METHODS

R-PIA and CPCA were obtained from Research Biochemical Inc. Zinc powder and cadmium acetate were from Merck. LPS from *Escherichia coli* (0127:B8 and 055:B5), sodium nitrite, DMSO, sulfanilic acid, *N*-ethylenediamine dihydrochloride, NECA, CHA and other chemicals were purchased from the Sigma Chemical Co. LPS was prepared in pyrogen-free 0.9% NaCl. NECA, *R*-PIA, CPCA and CHA were prepared as stock solutions in 4% DMSO in saline and diluted to required concentrations with saline.

Injection of animals. Male Swiss mice weighing 20–28 g, which had fasted overnight (18–24 hr) but been allowed free access to water, received a single injection of LPS (0127:B8, 5 mg/kg, i.p.). Control

† Corresponding author: Dr. S. Moochhala, Department of Surgery, National University of Singapore, Singapore 0511. Tel. (065) 7724232; FAX (065) 7778427.

‡ Abbreviations: NO, nitric oxide; LPS, lipopolysaccharide; NO_x^-, nitrates and nitrites; *R*-PIA, *R*-phenylisopropyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; CPCA, 5′-(*N*-cyclopropyl)carboxamidoadenosine; CHA, N^6-cyclohexyladenosine; NOS, nitric oxide synthase; iNOS, inducible NOS; cNOS, constitutive NOS; TNF, tumour necrosis factor; and IL-1, interleukin-1.

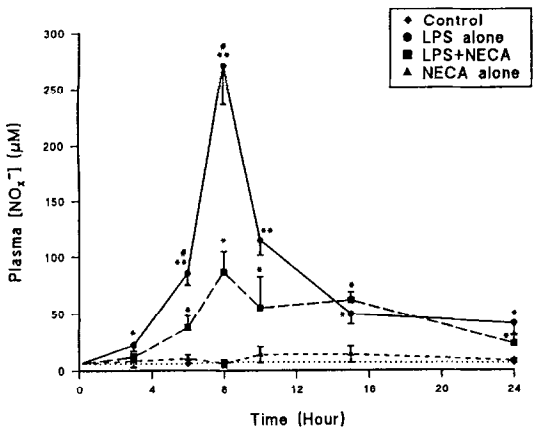

Fig. 1. Time course of plasma NO_x^- concentrations in LPS-treated mice. Plasma NO_x^- concentrations in control, LPS-treated, LPS- and NECA-treated, and NECA-treated mice are shown. Results are expressed as means ± SEM (N = 4). Key: (*) $P < 0.01$ and (**) $P < 0.001$ vs control; and (#) $P < 0.05$ vs LPS + NECA

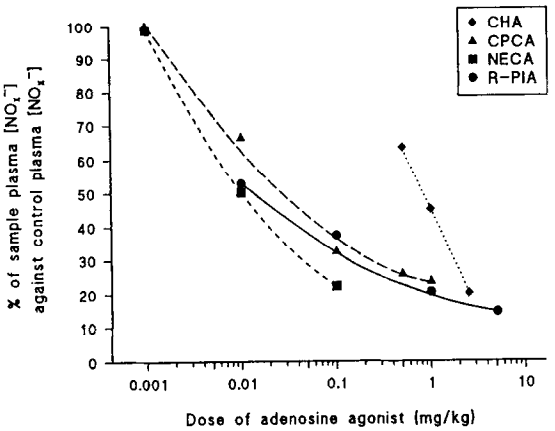

Fig. 2. Inhibitory effects of the adenosine agonists *R*-PIA, NECA, CHA and CPCA on the production of plasma NO_x^- in LPS-treated mice. Mice were killed 8 hr after LPS injection. The ED_{50} values ranged from 0.01 to 0.85 mg/kg. The sample plasma $[NO_x^-]$ is expressed as a percentage of the control (LPS-treated) plasma $[NO_x^-]$, and each point represents the mean (N = 4). Plasma $[NO_x^-]$ of the control (LPS-treated) was 270.75 ± 34.3 μM (N = 4).

animals received an appropriate volume of 0.9% NaCl. The animals were killed at 3, 6, 8, 10, 15 and 24 hr after the LPS injection. To study the effects of the adenosine receptor agonists, different groups of animals received different adenosine agonists i.p. 1 hr before the injection of LPS. Mice were killed 8 hr after LPS administration, the time corresponding to the plasma NO_x^- peak.

Plasma collection. Mice were anesthetized with ether and bled from their axillary vessels. The blood was collected in heparinized tubes, which were then centrifuged at 5000 *g* for 10 min in a microfuge. The plasma was transferred into clean tubes and stored at −70° until used.

Measurement of plasma NO_x^-. NO_2^- and NO_3^- concentrations in plasma were determined based on the Greiss reaction, in which NO_2^- reacts with 1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride in 5% H_3PO_4 to form a chromophore absorbing at 540 nm [13]. Copperized cadmium prepared according to the method of Rockett *et al.* [14] was used to reduce all the NO_3^- in the plasma to NO_2^-. Plasma NO_x^- concentration was obtained by comparing the absorbance value with a nitrite standard curve.

Statistics. All data are presented as means ± SEM. Statistical significance of differences between groups was determined by an unpaired Student's *t*-test. A probability (P) value < 0.05 was taken to indicate statistical significance.

RESULTS AND DISCUSSION

Mice injected with saline (the control) had a plasma NO_x^- concentration of 7.9 ± 1.2 μM (N = 8). Injection of the mice with LPS resulted in a time-dependent increase in the concentration of NO_x^- in the plasma, which was significantly higher than the control value after 6 hr, reached a peak of about 38 times the control value after 8 hr, and thereafter declined slowly toward control levels by 24 hr (Fig. 1).

We investigated the effects of four adenosine agonists at various concentrations on plasma NO_x^- levels in LPS-treated mice. The animals received the adenosine agonists 1 hr before the injection of LPS, and the animals were killed 8 hr after the LPS injection, the time corresponding to the NO_x^- peak. The agonists were chosen for their different affinities for adenosine A_1 and A_2 receptor subtypes. NECA has equal affinity for both A_1 and A_2 receptors, *R*-PIA and CHA have higher affinity for the A_1 receptor, while CPCA is specific for the A_2 receptor [15]. Injection of an adenosine agonist alone did not change the basal NO_x^- levels (7.9 ± 1.2 μM). Each agonist inhibited NO_x^- production in a dose-dependent manner, with ED_{50} values ranging from 0.01 to 0.85 mg/kg (Fig. 2). NECA ($A_1 = A_2$) and *R*-PIA ($A_1 > A_2$) were the most potent in reducing NO_x^- levels.

NECA was then chosen for further investigation. The time course of NO_x^- production showed that the inhibitory effect of NECA (0.1 mg/kg) appeared early (6 and 8 hr) after LPS injection but was no longer effective after approximately 10–15 hr (Fig. 1). This result suggests that the adenosine agonists can substantially reduce peak NO_x^- concentrations. Injection of NECA (0.1 mg/kg) 1, 2 and 3 hr after LPS injection reduced the NO_x^- levels to 31, 53 and 88%, respectively, of the LPS control value (270.75 ± 34.3 μM, N = 4). Reduction of NO_x^- levels by NECA appeared to be less effective the longer the interval of treatment after LPS injection. Therefore, the effect of NECA on NO release seemed to be an early event, possibly through the release of certain cytokines such as TNFα.

The order of inhibitory potency for NO_x^-

production was NECA ≥ *R*-PIA > CPCA > CHA, which is not specific for either A_1 or A_2 receptors. This is similar to the reports for adenosine inhibition of human monocyte TNF production [11]. Thus, there is a possibility that adenosine receptors other than A_1 and A_2 may be involved in the anti-inflammatory process. The possible involvement of other receptor subtypes, such as the A_3 receptor, remains to be determined using selective ligands [16].

Release of NO by iNOS is enhanced after immunological stimulation. NO is released as part of the host defence mechanism, as it is cytotoxic or cytostatic for tumour cells and invasive organisms. However, recent findings show that the release of NO may have other biological consequences, including pathological vasodilation and tissue damage [17]. Our results show that injection of the adenosine agonist before the treatment of mice with LPS will reduce mortality. Only one out of the six mice injected intraperitoneally with LPS (055:B5, 100 mg/kg) survived after 72 hr. However, mortality rates decreased if *R*-PIA (5 mg/kg, i.p.) was injected 1 hr beforehand, with four out of the six mice injected with the same dosage of LPS surviving after 72 hr. All the mice injected with saline or *R*-PIA alone survived after 72 hr. *R*-PIA rather than NECA was chosen because the latter alone has inherent toxic effects (unpublished observation).

There are a few possible explanations for the inhibitory and protective effects of adenosine agonists on LPS-induced changes. The agonists may be inhibiting the synthesis of cytokines such as TNF with consequent inhibition of the induction of the NOS, or they may be directly inhibiting the expression of this enzyme and thus blocking the release of NO from effector cells such as the macrophages, neutrophils and endothelial cells. The inhibitory effect may also be a direct effect of adenosine agonists since binding to the A_2 receptor leads to an increase in 3′,5′-cyclic adenosine monophosphate (cAMP), which activates various cellular functions. Increased levels of cAMP have been shown to prevent PMN adherence to endothelial cells as well as to decrease superoxide production and phagocytic activity, resulting in some possible anti-inflammatory effects [18, 19]. However, whether one or all the mechanisms suggested above is correct has yet to be established. Current study (unpublished observations) in our laboratory suggests that the adenosine agonists may be inhibiting the induction of iNOS mRNA.

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